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Decreased Nitric Oxide Production in Chronic Viral Hepatitis B and C

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Nitric oxide is a free radical gas molecule which may be implicated in antiviral defense. However, there is no information about its possible role in chronic viral hepatitis B and C. In this study we have analyzed the serum levels of NO_2^- (as an index of nitric oxide generation) from patients with chronic viral hepatitis B and C and relationship of same with the response to interferon therapy. Serum samples were analysed from 61 patients with chronic hepatitis B, 60 patients with chronic hepatitis C, 11 with chronic liver disease of nonviral origin, and 23 healthy controls. Levels of NO_2^- were statistically higher in healthy controls ($P < 0.001$) than in patients with chronic liver disease. No relation was found between NO_2^- and viremia or response to interferon therapy in patients with chronic hepatitis B. In contrast in chronic hepatitis C, responder patients had significantly higher NO_2^- than nonresponders ($P < 0.01$). With respect to the relation between NO_2^- levels and liver damage, patients with cirrhosis had lower NO_2^- levels than the rest of the patients ($P < 0.001$). In conclusion, patients with chronic viral hepatitis have low serum NO_2^- levels. ***J. Med. Virol. 51:326–331, 1997.***

KEY WORDS: hepatitis C virus; hepatitis B virus; interferon; nitrite levels; antiviral

INTRODUCTION

Nitric oxide (NO) is a free radical gas molecule which mediates several physiological functions, including host response to infections [Lowenstein et al., 1994; Nathan and Xie, 1994]. NO is produced through enzymatic conversion of L-arginine to L-citrulline by nitric oxide synthases [Nathan, 1992]. NO has a half life of seconds; it is converted in vitro and in vivo into nitrite (NO_2^-) and nitrate (NO_3^-) [Marletta et al., 1988; Tracey et al., 1995]. Nitric oxide synthases are encoded in three related genes in different tissues. Endothelial and neuronal enzymes are expressed constitutively [Lamas et al., 1992; Snyder, 1992], while macrophages and other cell types (including hepatocytes) have an inducible form of the nitric oxide synthase [Xie et al., 1992; Geller et al., 1993].

Nitric oxide is one of the principal mechanisms of macrophage cytotoxicity for tumor cells, bacteria, protozoa, helminths, and fungi [Nathan and Hibbs, 1991; Anggard, 1994]. There is also evidence of an antiviral activity of NO against herpes simplex, ectromelia, and vaccinia viruses both in vitro and in vivo [Karupiah et al., 1993; Kenneth, 1993]. However, to our knowledge, there have been no reports about the possible role of NO in chronic viral hepatitis B and C.

On the other hand, alpha interferon (IFN-alpha) has been used for the treatment of chronic hepatitis B and C. Approximately 40 to 50% of the patients treated responded to this therapy [Davis et al., 1989; Di Bisceglie et al., 1989; Perrillo et al., 1990; Carreño et al., 1993]. Several predictive factors of response to IFN alpha in chronic viral hepatitis have been identified [Brook et al., 1989; Rumi et al., 1994]. However, no reports have been published concerning the possible relation between NO production and response to IFN.

For the above reasons, in this study we have analyzed the serum levels of NO_2^- from patients with chronic viral hepatitis B and C and its relationship with the response to IFN therapy in those patients.

PATIENTS AND METHODS

Patients

Basal serum samples from 132 patients with histologically proved chronic hepatitis were analyzed in this study. Of these patients, 61 patients had a chronic hepatitis B (26, HBeAg+ with HBV-DNA detectable by dot-blot hybridization, and 35, anti-HBe+ with viral DNA detectable by PCR), 60 had a chronic hepatitis C (all of them anti-VCH positive and with serum HCV-

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TABLE I. Basal Features of the Patients

	Hepatitis B	Hepatitis C	Chronic hepatitis of nonviral origin
Number of patients	61	60	11
Age (years)[a]	36.6 ± 12.3	41.9 ± 13.1	39.8 ± 10.3
Gender (male/female)	46/15	38/22	7/4
ALT (IU/l)[a]	196.0 ± 287.3	170.6 ± 112.4	176.5 ± 141.5
Liver histology	27 CAH	28 CAH	3 autoimmune CAH
	7 CPH	7 CPH	1 alcoholic CAH
	7 Ci	17 Ci	4 primary biliary cirrhosis
	20 MLC	8 MLC	3 ductopenia CAH
Known duration of the disease (months)	53.7 ± 48	71.8 ± 79.3	62.2 ± 38
Epidemiology			
Transfusion	2	20	—
Parenteral	1	10	—
Sexual	9	—	—
Unknown	49	30	11

[a]Expressed as mean ± SD.
CAH: chronic active hepatitis; CPH: chronic persistent hepatitis; Ci: active cirrhosis; MLC: minimal liver changes.

RNA), and 11 had a chronic liver disease of nonviral origin (4 with primary biliary cirrhosis, 3 with autoimmune hepatitis, 1 with alcoholic chronic hepatitis, and 3 with ductopenia). Clinical features of the patients are shown in Table 1.

Forty-one of the 61 patients with chronic hepatitis B were included in differing IFN alpha trials with doses ranging from 1.5 MU/m^2 of body surface to 18 MU/m^2 of body surface for a period of 4 to 12 months. Of these patients, 18 were responders (HBV-DNA negative and normal ALT levels at the end of therapy) and the rest nonresponders. With respect to the patients with chronic hepatitis C, 54 of the 60 patients were also treated with IFN alpha, with doses ranging from 1.5 MU/m^2 of body surface to 10 MU/m^2 of body surface, for a period of 6 to 12 months. Fourteen of the treated patients were long-lasting responders (with normal ALT values for at least 6 months after the end of therapy). In treated patients the serum sample analyzed was taken before the start of the therapy.

As study controls, serum samples were also analyzed from 23 healthy controls without viral markers and with normal liver function tests.

All serum samples were aliquoted and immediately frozen after phlebotomy. Each aliquot was used only once for NO_2^- measurement.

Detection of Viral Markers in Serum

The detection of viral infection markers and the assessment of liver function tests were performed on the same serum sample for each patient.

HBsAg, HBeAg, and anti-HBe were detected by commercial radioimmunoassays (Abbott Laboratories, North Chicago, IL).

Detection of HBV-DNA by dot hybridization was carried out as described [Berninger et al., 1982]. HBV-DNA concentration was measured in a Radioanalytical Imaging System (Ambis, San Diego, CA), using as standards serial dilutions of cloned HBV-DNA of known concentration. Amplification of HBV-DNA by polymerase chain reaction (PCR) was carried out using conserved primers from the PreC/C region of the viral genome, as described [López-Alcorocho et al., 1994].

Anti-HCV was determined by ELISA (Ortho Diagnostic System, Raritan, NJ) and confirmed by RIBA II (Ortho). HCV-RNA was detected qualitatively by the polymerase chain reaction using primers from 5′ noncoding region of the HCV genome [Castillo et al., 1992].

Liver function tests were analyzed by standard methods (Smac 20, Technicon, New York, NY).

Detection of Serum Nitrite Levels

As an index of NO generation, NO_2^- levels were measured [Scott-Burden et al., 1992; Keisuke et al., 1995]. For the measurement of nitrite (NO_2^-) levels, serum samples were diluted 1:3 in distilled water, and 100 μl of the dilution were deproteinized by treatment with 35% sulfosalicylic acid and centrifuged before analysis. NO_2^- levels were tested in the supernatants by the Griess reaction, as described [Green et al., 1982].

The Griess reagent consisted of one part 0.003 M napthylenediamine dihydrochloride and one part of 0.05 M sulfanilamide in 0.3 M phosphoric acid (obtained from Sigma Chemical CO., St. Louis, MO), mixed and kept chilled. The color of the product was developed after incubation in a water bath at 60°C for 1h. The absorbance of the product dye was measured at 540 nm on visible spectrophotometry. Serial dilutions of $NaNO_2^-$ of known concentration (from 0.1 μM to 50 μM) were used as standards.

Statistical Analysis

NO_2^- levels are presented as mean values (mean ± standard deviation) from 7 independent experiments.

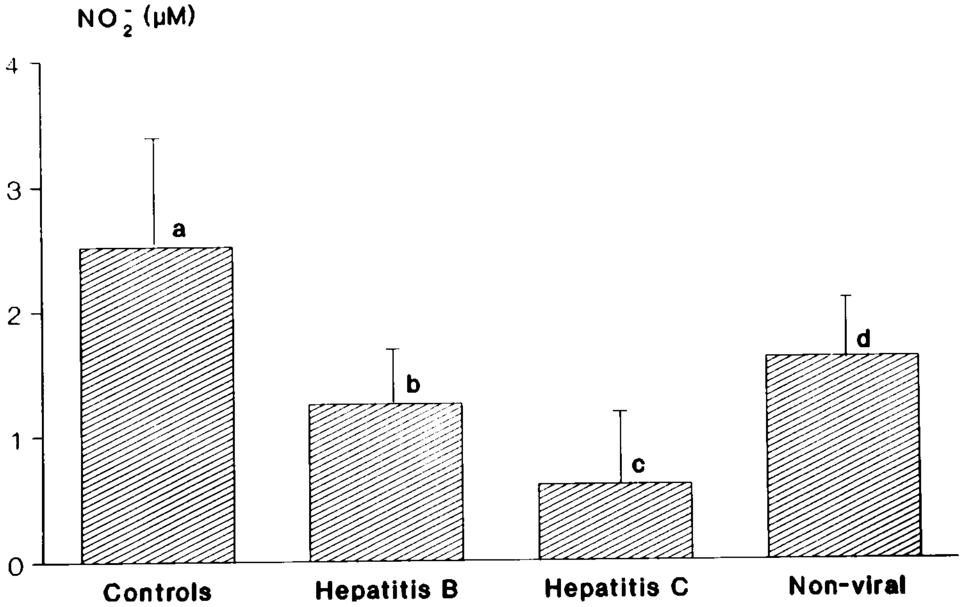

Fig. 1. Serum NO_2^- levels in healthy controls and patients with chronic liver disease. **a** vs. **b, c, d,** $P < 0.001$, **b** vs. **c, d,** $P < 0.05$, **c** vs. **d,** $P < 0.001$.

Difference significance was assessed by analysis of variance and the Tukey test, by statistical package Stat Graphics™, for single and multiples comparisons. The Student's t-test were used when applicable, and sample correlations were used to correlate NO_2^- with HBV-DNA concentration and alanine amino transferase (ALT) levels. A P value of less than 0.05 was considered statistically significant.

RESULTS

NO_2^- levels were statistically higher in healthy controls (2.5 ± 0.9 μM) than in patients with chronic hepatitis B (1.2 ± 0.4 μM), chronic hepatitis C (0.6 ± 0.5 μM), or chronic hepatitis of nonviral origin (1.6 ± 0.4 μM) (Fig. 1).

NO_2^- Levels in Patients With Chronic Hepatitis B

In an attempt to correlate NO_2^- and viremia levels, patients with chronic hepatitis B were divided into HBeAg+ (with high levels of viremia demonstrated by the detection of HBV-DNA in serum by dot-blot hybridization) and anti-HBe (low viremic patients with serum viral DNA detectable only by PCR). No statistical differences in NO_2^- levels were found between HBeag and anti-Hbe patients (1.3 ± 0.8 μM and 1.13 ± 0.34 μM respectively). Furthermore, no statistical correlation was observed between NO_2^- levels and HBV-DNA concentration in HBeAg positive patients (data not shown). These results show that there is no correlation between NO_2^- and the degree of HBV replication.

With respect to the relation between NO_2^- levels and the outcome of IFN therapy in HBV chronic carriers, no statistical differences were found between responders (1.23 ± 0.7 μM) and nonresponders (1.27 ± 0.76 μM), demonstrating that in these patients, NO_2^- is not a predictive factor of response to IFN.

NO_2^- Levels in Patients With Chronic Hepatitis C

All patients with chronic hepatitis C had HCV-RNA in serum. Patients with chronic hepatitis C who did not respond to IFN α treatment (abnormal ALT after therapy) had significantly lower levels ($P < 0.01$) of NO_2^- (0.32 ± 0.3 μM) than those patients who normalized the ALT during treatment and remained the same during follow-up (0.81 ± 0.5 μM). What is more, the level of NO_2^- in nonresponder patients was also significantly lower than that in patients who had normalized ALT during treatment but suffered an increase in ALT levels during follow-up (0.32 ± 0.3 μM vs. 0.82 ± 0.5 μM, $P < 0.001$).

NO_2^- Levels and Liver Damage in Patients With Chronic Viral Hepatitis

No correlation between ALT and NO_2^- levels was found in patients with chronic hepatitis B or C (data not shown). NO_2^- levels were compared with the histological diagnosis of the patients included in the study. A relation between the NO_2^- levels and the severity of the histological damage was observed. Accordingly, patients with liver cirrhosis had significantly lower levels of NO_2^- (0.45 ± 0.4 μM) than those with chronic active hepatitis (0.93 ± 0.8 μM) ($P < 0.0001$), chronic persistent hepatitis (1.05 ± 0.4 μM) ($P < 0.0001$), or minimal changes (1.29 ± 0.19 μM) ($P <$

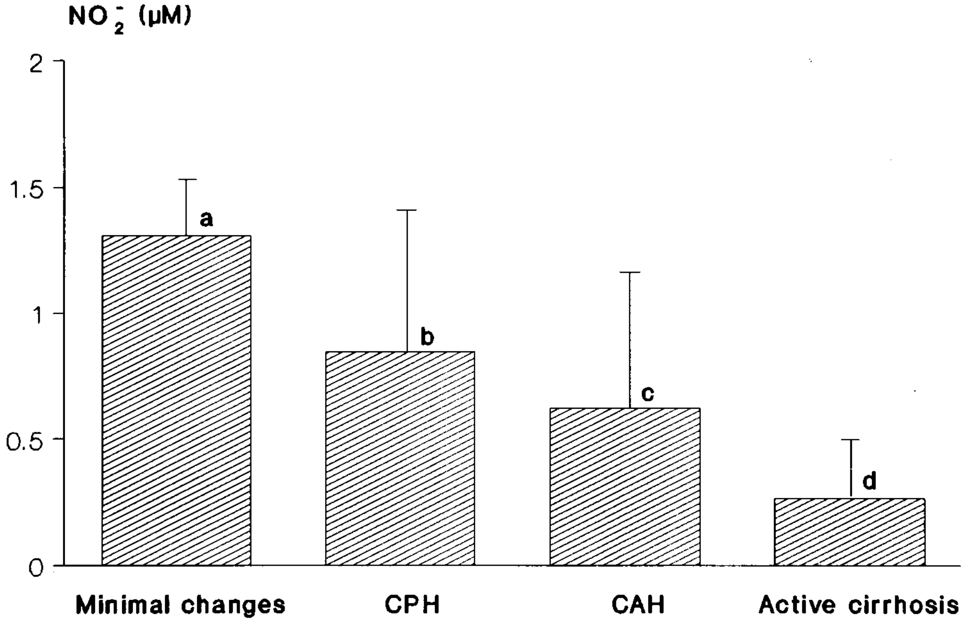

Fig. 2. Serum NO_2^- levels and histological diagnosis in patients with chronic hepatitis C, active cirrhosis (Ci) (0.26 ± 0.3 μM), chronic active hepatitis (CAH) (0.6 ± 0.6 μM), chronic persistent hepatitis (CPH) (0.85 ± 0.6 μM), and minimal changes (MLC) (1.3 ± 0.2 μM). Data expressed as mean ± standard deviation. **d** vs. **a, b, c,** $P < 0.05$, **c** vs. **a,** $P < 0.005$, **b** vs. **a,** $P < 0.05$.

0.0001). These differences in NO_2^- levels cannot be attributable to differences in the known duration of the liver disease since no statistical differences in this parameter was found between patients with cirrhosis (82.3 ± 92 months), chronic active hepatitis (57.6 ± 52 months), chronic persistent hepatitis (66 ± 51 month), or minimal changes (65 ± 59 months). In order to determine whether this tendency remained present in chronic hepatitis due to HCV or to HBV, the histological diagnosis was compared with the NO_2^- levels within each type of viral hepatitis. Among patients with chronic hepatitis C, those with a more severe liver disease had significantly lower levels than the other patients (Fig. 2). No statistical difference was found in the known duration of the disease between patients with liver cirrhosis (72 ± 64 months), chronic active hepatitis (76 ± 67 months), chronic persistent hepatitis (74 ± 60 months), and minimal liver change (40.4 ± 36 months). By contrast, in patients with chronic hepatitis B, NO_2^- levels were similar (without statistical differences) in patients with liver cirrhosis (1.05 ± 0.49 μM), chronic active hepatitis (1.30 ± 0.75 μM), chronic persistent hepatitis (1.14 ± 0.34 μM), and minimal liver changes (1.19 ± 0.31 μM). Again, no statistical differences were found on the carriage time in patients with liver cirrhosis (60 ± 42 months), chronic active hepatitis (42.2 ± 45 months), chronic persistent hepatitis (41.5 ± 33 months), or minimal liver changes (82 ± 64 months).

Cholesterol Levels and Serum NO_2^- Levels

Cholesterol may influence NO production [Minor et al., 1990; Wang et al., 1994], and in consequence, these levels were measured in the study patients. No differences in cholesterol were found among patients with chronic hepatitis B (186 ± 46 mgr/dl; range 116–237), chronic hepatitis C (177 ± 3 mgr/dl; range 126–226), chronic hepatitis of nonviral origin (209 ± 33 mgr/dl; range 155–223), and healthy controls (182 ± 26 mgr/dl; range 138–230).

DISCUSSION

Nitric oxide has a variety of biological functions in various tissues [Moncada et al., 1991]. There is also evidence as to its antiviral activity against herpes simplex, ectromelia, and vaccinia viruses [Karupiah et al., 1993; Kenneth, 1993; Lowenstein et al., 1996]. To our knowledge, there are no reports about its possible role in chronic viral hepatitis B and C. In this study, we have measured serum NO_2^- levels as an index of NO generation in patients with chronic viral hepatitis B and C, in comparison with patients with chronic liver disease of nonviral etiology and with a group of healthy controls with normal liver function tests.

Patients with chronic liver disease (of either viral or nonviral origin) were found to have significantly lower levels of NO_2^- than the healthy control group. However, when patients with chronic hepatitis were divided according to the etiology of the disease, patients with chronic viral hepatitis had statistically lower serum NO_2^- levels than patients with chronic hepatitis of nonviral origin, patients with chronic hepatitis C having the lower levels.

The reason for the production of NO in patients with chronic viral hepatitis is not clear. Tumor necrosis factor alpha, Interleukine 1Beta, and gamma interferon are inducers of nitric oxide synthase of both macrophages and hepatocytes [Nusselr et al., 1992; Summersgill et al., 1992], but the levels of these are increased in patients with chronic hepatitis B and C [Yoshioka et al., 1989; Sheron et al., 1991; Quiroga et al., 1994]. Therefore, the low NO_2^- generation shown in these patients cannot be attributed to a low production of these citokines. However, a low sensitivity to these

factors in these patients can not be discarded. Furthermore, cholesterol levels, which may influence NO_2' generation [Green et al., 1982; Minor et al., 1990] were similar in patients with liver disease and healthy controls.

Another interesting observation was that NO_2^- levels were statistically higher in patients with chronic hepatitis C who had responded to IFN alpha therapy than in nonresponder patients, suggesting that nitric oxide generation may be a predictive factor of response in this type of patient, although it should be proved in future studies. On the other hand, NO_2^- levels were similar in responder and nonresponder patients with chronic hepatitis B. These findings suggest a different role of nitric oxide in chronic hepatitis B and C which may or may not be related to biological differences, or differences in the pathogenical mechanism of the liver injury for both viruses. These hypotheses must be tested in future research.

Finally, we have observed that NO_2^- levels in patients with chronic viral hepatitis are statistically lower in cirrhotic patients than in those with chronic active or chronic persistent hepatitis, or with minimal changes. Furthermore, when these data were analyzed in greater detail, it was found that the differences in NO_2^- levels were present only in the patients with chronic hepatitis C, since no correlation between NO_2^- levels and liver damage was found in patients with HBV infection. This finding once again points up that nitric oxide production may have a different pathogenic role in HBV and HCV chronic infection. These differences in NO_2^- in patients with distinct histological diagnoses are not due to differences in the known duration of the disease.

With respect to the cirrhotic patients, the results differ from those of Guarner et al. [1993], who have found high serum nitrite and nitrate levels in cirrhotic patients. However, most of the patients studied by Guarner and his colleagues were endotoxemic, and endotoxemia is a well known inducer of NO_2^- production [Knowles et al., 1990; Evana et al., 1992]. In contrast, none of the cirrhotic patients analyzed in our study had decompensated cirrhosis or endotoxemia. Moreover, only a small proportion (6/51, 11.7%) of the cirrhotic patients studied by Guarner et al. had a liver disease due to a chronic HBV infection.

In conclusion, the data show that patients with chronic viral hepatitis have low NO_2^- levels in serum which can not be attributed to a lower cytokine production, differences in serum cholesterol levels, or differences in the known duration of the disease. The question of whether HBV and HCV are able to inhibit nitric oxide production or that the maintenance of the hepatitis B and C carrier state is due to a preexistent deficit in NO production should be tested in future studies.

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